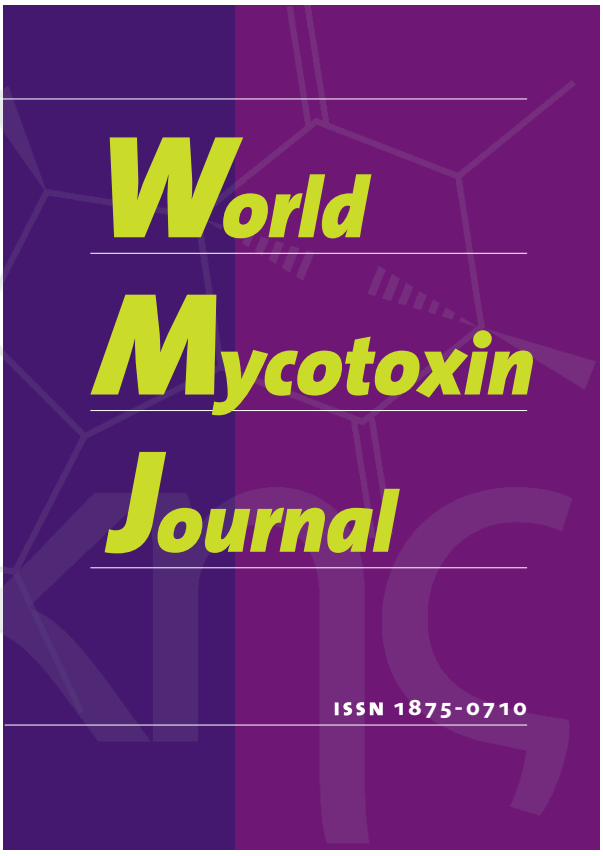

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Association between high aflatoxin B_1 levels and high viral load in HIV-positive people

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Abstract

Since both aflatoxin and the human immunodeficiency virus (HIV) cause immune suppression, chronic exposure to aflatoxin in HIV-positive people could lead to higher levels of virus replication. This study was conducted to examine the association between aflatoxin B_1 albumin adduct (AF-ALB) levels and HIV viral load. Antiretroviral naive HIV-positive people (314) with median CD4 count of 574 cells/µl blood (mean ± standard deviation = 630±277) were recruited in Kumasi, Ghana. Sociodemographic and health data, and blood samples were collected from participants. The plasma samples were tested for AF-ALB and HIV viral load. Univariate logistic regression analysis was conducted using viral load (high/low) as the outcome and AF-ALB quartiles as exposure. Multivariable logistic regression analysis was performed between quartile AF-ALB, viral load and CD4 adjusting for sex, age, and year of HIV diagnosis. Both univariate and multivariable logistic regression showed that viral load increased as AF-ALB levels increased. By univariate analysis, high viral load was 2.3 times more likely among persons in the third AF-ALB quartile (95% confidence interval (CI): 1.13, 4.51), and 2.9 times more likely among persons in the fourth AF-ALB quartile (CI: 1.41, 5.88), compared to persons in the first quartile. In the multivariable model, persons in the fourth AF-ALB quartile were about 2.6 times more likely to have high viral loads than persons in the first quartile (CI: 1.19-5.69). When AF-ALB and viral load were log transformed and linear regression analysis conducted, the univariate linear regression analysis showed that for each pg/mg increase in AF-ALB, viral load increased by approximately 1.6 copies/ml (P=0.0006). The association was marginally significant in the adjusted linear regression model (i.e. for each pg/mg increase in AF-ALB, the mean viral load increased by approximately 1.3 copies/ml, P=0.073). These data show strong and consistent increases in HIV viral load with increasing AF-ALB levels. Since the median and mean CD4 were greater than 500 cells for participants in each AF-ALB quartile, the results indicate that the immune modulating and virus transcription effects of aflatoxin may occur quite early in HIV infection, even while the CD4 count is still above 500, resulting in higher viral loads.

Keywords: aflatoxin B_1 albumin adducts, HIV viral load, Ghana

1. Introduction

Aflatoxins, a group of extremely toxic and carcinogenic metabolites produced by the common fungi *Aspergillus flavus* and *Aspergillus parasiticus,* have been shown to be immunosuppressive in a number of animal and human studies (Bondy and Pestka, 2000; Gabal and Azzam, 1998; Jiang *et al.*, 2005; Meissonnier *et al.*, 2008; Pier, 1986; Turner *et al.*, 2003). The toxin builds up in staple food crops mainly during post-harvest handling and storage under hot and humid climatic conditions (Hell *et al.*, 2000). In Ghana, as well as other sub-Saharan African countries, and developing tropical countries of Southeast Asia and Latin America, staple food crops such as groundnuts, maize, rice and other cereals are often contaminated with levels of aflatoxin that far exceed the 30 µg/kg considered tolerable in food for human consumption by the FAO/WHO/UNICEF Protein Advisory Board (Awuah and Kpodo, 1996; Begum and

Samajpati, 2000; Carvajal and Arroyo, 1997; Freitas and Brigido; Hell *et al.*, 2000; JECFA, 1998).

There is strong evidence to show that low level aflatoxin exposure can increase susceptibility to infectious diseases in different animal species, such as dysentery in swine and *Salmonella* and fowl adenovirus seroptype-4 infections in chickens (Boonchuvit and Hamilton, 1975; Joens *et al.*, 1981; Shivachandra *et al.*, 2003). Low level aflatoxin exposure has also been shown to reactivate chronic *Toxoplasma gondii* infection in mice (Venturini *et al.*, 1996), to reduce the antibody response to vaccines in animals (Gabal and Azzam, 1998; Gabal and Dimitri, 1998; Schivachandra *et al.*, 2003) and to decrease the cell-mediated immune response to a vaccine antigen in pigs (Meissonnier *et al.*, 2008). A study conducted in Gambian children reported that immunogloubulin A in saliva may be reduced by dietary levels of aflatoxin (Turner *et al.*, 2003).

Since both aflatoxin and the human immunodeficiency virus (HIV) are immunosuppressive agents, we hypothesised that aflatoxin exposure may adversely influence the pattern of HIV infection and lead to faster progression to acquired immune deficiency syndrome (AIDS) in infected individuals. Sub-Saharan Africa has the largest HIV/AIDS epidemic worldwide (UNAIDS, 2012) and millions of HIV-infected people in this region of the world are likely chronically exposed to aflatoxin in their diets (CAST, 2003; IARC, 2002; Jiang *et al.*, 2008). Previously, we investigated the possible interaction of aflatoxin and HIV on immune suppression by comparing immune parameters in HIV-positive and aged-matched HIV-negative Ghanaians with high and low aflatoxin B_1 albumin adduct (AF-ALB) levels (Jiang *et al.*, 2008). We found that among both HIV-positive and -negative participants, high AF-ALB was associated with lower perforin expression on CD8+ T-cells and that HIV-positive participants with high AF-ALB had significantly lower percentages of CD4+ T regulatory cells (Tregs), naive CD4+ T cells, and B-cells, compared to those with low AF-ALB. Thus, high AF-ALB appeared to intensify some HIV-associated changes in T-cell phenotypes and B-cells in HIV-positive individuals. We also found that the mean AF-ALB level was significantly higher for the HIV-positive compared to the HIV-negative group and that among HIV-positive participants, those with high AF-ALB were significantly more likely to have higher HIV viral loads than those with low AF-ALB (Jolly *et al.*, 2011). Since only 155 HIV-positive participants were recruited in the previous study and these participants were at different stages of HIV infection (38% had CD4 below 200 and were on antiretroviral therapy) it was difficult to extricate if observed associations were as a result of HIV infection, HIV treatment, other HIV/AIDS accompanying conditions or opportunistic infections. Thus, we recruited a larger sample of antiretroviral therapy (ART) naive HIV-positive individuals with high CD4 count (median = 574 cells/µl of blood; mean ± standard deviation = 630±277) and examined the association between AF-ALB and HIV viral load.

2. Methods

Study site, study participants and data collection

A cross-sectional study among ART naive HIV-positive adults (≥18 years) with median CD4 counts of 574 cells/µl blood was conducted in two hospitals (Kumasi South Regional and Bomso Hospitals) in Kumasi, Ghana from February to May 2009. The Kumasi South Regional Hospital (KSRH) is located between three cities (Atonsu, Agogo and Chirapatre) in the Ashanti Region and provides services to 56 communities, which consist of approximately 400,000 people. Bomso Hospital (BH) is a specialised 163 bed private hospital in Kumasi that has a comprehensive HIV care, treatment and support programme. BH is in close proximity to, and works closely with, KSRH. Approval for the study was obtained from the Institutional Review Board at the University of Alabama at Birmingham (UAB) and the Committee on Human Research, Publications and Ethics, School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi. After informed consent was obtained, a standardised interviewer-administered questionnaire was used to obtain demographic, health-related and food consumption information from participants. The interview was conducted in private rooms at the hospitals. The medical records of patients were reviewed to obtain clinical information including any clinical diagnoses, medications prescribed, HIV diagnosis date and CD4+ count. A 20 ml blood sample was collected from each participant in EDTA vacutainer tubes by trained clinic staff. After centrifugation the plasma was aspirated, stored frozen at -80 °C and shipped to UAB for determination of AF-ALB levels and HIV viral load.

Determination of aflatoxin B_1-lysine adducts

Plasma aflatoxin B_1 (AFB_1)-lysine adducts, reflecting aflatoxin exposure in the previous 2-3 months, were measured by a modified HPLC fluorescence method (Qian *et al.*, 2013). Briefly, plasma samples (150 µl) were digested by Pronase (Calbiochem, San Diego, CA, USA) and loaded onto an Oasis Max cartridge from Waters Co. (Milford, MA, USA). The cartridges were sequentially washed, and eluted with 2% formic acid in methanol. The eluents were evaporated to dryness and reconstituted with 150 µl 10% methanol before HPLC analysis. HPLC analysis was carried out on an 1100 liquid chromatography system (Agilent Technologies, Wilmington, DE, USA). Chromatographic separation was performed on an Agilent C18 column (5 µm particle size, 250×4.6 mm). The mobile phase consisted of 20 mM ammonium phosphate monobasic (pH 7.2) and methanol in a linear gradient profile. The concentration of AFB_1-lysine adducts was monitored at wavelengths of 405

nm (excitation) and 470 nm (emission). Peaks representing authentic AFB_1-lysine adduct standard or from positive samples were eluted with a retention time of approximately 12.7 min. The concentration of AFB_1-lysine adducts were adjusted by serum albumin level and reported as pg/mg albumin. The detection limit of this method is 0.4 pg/mg albumin.

Quantitative HIV-1 RNA assay for HIV viral load

The Roche COBAS Ampliprep/COBAS TaqMan HIV-1 Test, version 2.0 was used for quantitation of HIV-1 RNA in plasma of study participants (Roche Molecular Systems, Inc., Pleasanton, CA, USA) at the UAB Hospital Laboratory. The test was used to quantify HIV-1 RNA based on the co-amplification of two distinct regions of the HIV genome: LTR (long terminal repeat) and *gag*. The dual PCR target enhances the ability to quantify diverse HIV-1 samples, such as HIV-1 Group M subtypes, including circulating recombinant forms and HIV-1 Group O. The linear range is 20 to 10 million copies/ml. Testing is based on three major processes: automated specimen preparation to isolate HIV-1 RNA; automated reverse transcription of target RNA to generate complementary DNA (cDNA); and automated simultaneous PCR amplification of target cDNA and detection of cleaved dual-labelled oligonucleotide detection probe specific to the target. Quantitation of HIV-1 viral RNA was performed using a Quantitation Standard that compensates for effects of inhibition and controls the preparation and amplification processes. This test is approved by the United States Food and Drug Administration.

Statistical analyses

We compared socio-demographic variables by sex using Chi-square tests. AF-ALB was divided into quartiles (1^{st} quartile = 0.20-4.97; 2^{nd} quartile = 4.98-10.63; 3^{rd} quartile = 10.64-20.27; 4^{th} quartile = 20.28-109.87 pg/mg albumin) and viral load divided into high (≥10,000 copies/ml) and low (≤9,999 copies/ml) levels. The viral load categorisation was based on published research that showed that HIV-positive people with viral loads below 10,000 copies/ml of blood did not progress to AIDS in over 9 years compared to those with viral loads ≥10,000 copies/ml (Rinaldo *et al.*, 1995). Univariate logistic regression was conducted using viral load as the outcome and AF-ALB quartiles as exposure with the lowest AF-ALB quartile as the reference category. Multivariable logistic regression analyses were also performed between quartile AF-ALB, viral load and CD4 adjusting for sex, age and year of HIV diagnosis. Both AF-ALB levels and HIV viral load were then log transformed and the association between AF-ALB and viral load examined by univariate linear regression analysis. Finally, multivariable linear regression analyses were performed between log AF-ALB, log viral load, CD4 and year of HIV diagnosis adjusting for sex and age. All analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). The significance level was set at $P<0.05$.

3. Results

Sociodemographic characteristics of the study population by sex

Of the 314 participants recruited for the study, 77% were females (Table 1). Although most participants (45.5%) were 30-39 years, a higher proportion of females (34%) than males (12.7%) were 18-29 years ($P=0.002$). The majority of participants were married (68%). Males were more likely than females to have attended high school (25.4% vs. 13.6%; $P=0.018$), to earn ≥50 Ghana cedis per month (1.4 Ghana cedis = approximately US$ 1.00 in 2009; 77.5% vs. 34.1%; $P=0.001$), and to have piped water in their homes (47.9% vs. 33.6%; $P=0.029$).

CD4 count, viral load, aflatoxin B_1 levels and year of HIV diagnosis for the study population

The median CD4 count for the study group was 574 cells/µl (mean ± standard deviation = 630±277 cells/µl) (Table 2). The median viral load was 32,550 copies/ml of blood (range = 19 (undetectable) to 820,000 copies/ml; mean ± standard deviation = 136,942±340,313). 68% of the study group had a viral load ≥10,000. AF-ALB ranged from 0.2-109.87 pg/mg albumin (median = 10.46 pg/mg albumin). Most participants (81%) were diagnosed with HIV between January 2008 and May 2009. The remaining participants were diagnosed as HIV-positive between 2006 and 2007.

Logistic regression of viral load (low vs. high) and quartile aflatoxin B_1 levels

The univariate logistic regression analysis showed that viral load increased as quartile AF-ALB levels increased ($P=0.0007$, data not shown). A high viral load was 2.3 times more likely among persons with AF-ALB in the third quartile vs. the first quartile (95% confidence interval (CI): 1.13, 4.51; $P=0.021$), and 2.9 times more likely among persons with AF-ALB in the fourth quartile vs. the first quartile (95% CI: 1.41, 5.88; $P=0.004$). Upon adjusting for CD4 count, sex, age, and year of HIV diagnosis, the trend of increase in viral load with increasing AF-ALB remained ($P=0.04$; Table 3). An adjusted high viral load was 2.6 times more likely among persons in the fourth AF-ALB quartile than the first quartile (95% CI: 0.19, 5.69; $P=0.02$), and 1.92 times more likely among persons in the third than the first quartile (95% CI: 0.92, 4.04; $P=0.08$ (Table 3). Similarly, upon adjusting for sex, age and year of HIV diagnosis, persons with CD4 count ≥500 cells/µl were 66% less likely to have high virus loads than those with CD4 count between 300 and 499 cells (95% CI: 0.19, 0.61; $P=0.001$; Table 3).

Table 1. Socio-demographic characteristics of the study population stratified by sex.

Characteristics	Overall, n (%)[1]	Female, n (%)	Male, n(%)	*P*-value[2]
	314 (100)	242 (77.1)	72 (22.9)	
Age (years)				**0.002**
18-29	90 (29.0)	81 (33.9)	9 (12.7)	
30-39	141 (45.5)	104 (43.5)	37 (52.1)	
≥40	79 (25.5)	54 (22.6)	25 (35.2)	
Marital status				0.298
Married	208 (68.2)	156 (66.7)	52 (73.2)	
Single	97 (31.8)	78 (33.3)	19 (26.8)	
Educational level				**0.018**
Primary or lower	103 (33.7)	87 (37.0)	16 (22.5)	
Junior High	153 (50.0)	116 (49.7)	37 (52.1)	
≥ High School	50 (16.3)	32 (13.6)	18 (25.4)	
Employment status				**0.015**
Employed	218 (71.7)	159 (68.2)	59 (83.1)	
Unemployed	86 (28.3)	74 (31.8)	12 (19.9)	
Monthly income (Ghana Cedis)[3]				**0.001**
<50	70 (47.0)	61 (56.0)	9 (22.5)	
50-100	63 (42.3)	39 (35.8)	24 (60.0)	
>100	16 (10.7)	9 (8.3)	7 (17.5)	
House has piped water				**0.029**
Yes	113 (36.9)	79 (33.6)	34 (47.9)	
No	193 (63.1)	156 (66.4)	37 (52.1)	
House has electricity				**0.009**
Yes	242 (79.1)	178 (75.7)	64 (90.1)	
No	64 (20.9)	57 (24.3)	7 (9.9)	
Religious affiliation				**0.099**
Christian	266 (87.2)	209 (88.9)	57 (81.4)	
Muslim/others	39 (12.8)	26 (11.1)	13 (18.6)	
Number of children 10-15 years in the household				0.068
≤ 1	110 (70.5)	89 (74.2)	21 (58.3)	
≥ 2	46 (29.5)	31 (25.8)	15 (41.7)	

[1] Not all participants have answered all the socio-demographic characteristics.
[2] Bold *P*-values are significant at $P<0.05$.
[3] 1.4 Ghana cedis were approximately equal to US$ 1.00 in 2009.

When we re-ran the analysis with 3 categories of viral load (<9,999, 10,000-100,000, and ≥100,000) in a proportional odds model, we obtained results similar to that obtained in the multivariable logistic model.

Linear regression between viral load and aflatoxin B_1 levels

Univariate linear regression analysis between viral load and aflatoxin showed that for each pg/mg increase in aflatoxin, viral load increases by approximately 1.6 copies/ml (P=0.0005, data not shown). However, after adjusting for age and sex in a multivariable linear regression model, the increase in viral load of 1.27 copies/ml obtained for each pg/mg increase in AF-ALB was only marginally significant (P=0.07; Table 4). Participants with lower CD4 counts at the time of enrolment had higher viral loads (Table 4). The mean difference in viral load among those diagnosed with HIV between 2006 and 2007 and between 2008 and 2009 is about 0.37 copies/ml. That is, patients diagnosed with HIV less than 2 years at the time of enrolment had higher viral loads than those diagnosed with HIV greater than 2 years (P=0.017).

4. Discussion

In this study we found significantly higher viral loads in HIV-positive people with higher AF-ALB levels by univariate and multivariable logistic, and univariate linear regression analyses. This consistent finding confirms our

Table 2. CD4, viral load, year of HIV diagnosis and aflatoxin B_1 levels of study participants.

Variable	Number (%)
CD4 count (cells/µl blood)[1]	
300-499	116 (39.3)
≥ 500	179 (60.7)
Viral load (copies/ml blood)[2]	
≤ 9,999	108 (32.1)
≥10,000	228 (67.9)
Year of HIV diagnosis	
2006-2007	55 (18.6)
2008-2009	240 (81.4)
Aflatoxin B_1 quartile[3]	**Range aflatoxin B_1 (pg/mg albumin)**
Quartile 1	0.20-4.97
Quartile 2	4.98-10.63
Quartile 3	10.64-20.27
Quartile 4	20.28-109.87

[1] CD4: median 574.3; mean ± standard deviation = 630±277 cells/µl blood.
[2] Viral load: median 32,550; mean ± standard deviation = 136,941.68 ±340,312.83 copies/ml blood.
[3] Aflatoxin B_1: median 10.46; mean ± standard deviation = 14.91±15.62 pg/mg albumin.

Table 3. Multivariable logistic regression model of viral load, aflatoxin quartiles and CD4 counts adjusting for sex, age and year of HIV diagnosis.

Variable	OR (95% CI) [a]	*P*-value [b]
Quartile aflatoxin (pg/mg albumin)		**0.04**
Q2 vs. Q1	1.04 (0.53, 2.05)	0.925
Q3 vs. Q1	1.92 (0.92, 4.04)	0.08 [c]
Q4 vs. Q1	2.60 (1.19, 5.69)	**0.02**
Sex		0.52
Male	Reference	
Female	0.80 (0.40, 1.58)	0.52
Age (years)		0.87
18-29	1.15 (0.55, 2.40)	0.71
30-39	0.97 (0.51, 1.86)	0.92
≥40	Reference	
CD4 count (cells/µl blood)		**0.00**
300-499	Reference	
≥500	0.34 (0.19, 0.61)	0.00
Year of HIV diagnosis		0.43
2006-2007	Reference	
2008-2009	1.30 (0.68, 2.48)	0.43

[a] OR = odds ratio; CI = confidence interval.
[b] Bold *P*-values are significant at $P<0.05$.
[c] Marginally significant.

Table 4. Multivariable linear regression of log viral load, log aflatoxin, CD4 count and year of HIV infection adjusting for age and sex.

Variable	Parameter estimate	*P*-value [a]
Log aflatoxin	1.27 (exp(0.242))	0.07 [b]
Sex		0.20
Male	Reference	
Female	0.60 (exp(-0.52))	0.20
Age in years		0.14
18-29	2.43 (exp(0.89))	0.05
30-39	1.78 (exp(0.58))	0.15
≥40	Reference	
CD4 count (cells/µl blood)		**<0.001**
300-499	5.40 (exp(1.69))	**<0.001**
≥500	Reference	
Year of HIV diagnosis		0.017
2006-2007	0.37 (exp(-0.99))	0.017
2008-2009	Reference	

[a] Bold *P*-values are significant at $P<0.05$.
[b] Marginally significant.

previous report of significant association between high AF-ALB and high HIV viral load (Jolly *et al.*, 2011). However, by recruiting ART naive HIV-positive people with high CD4 in this study, we were able to eliminate the effect HIV/AIDS-related clinical conditions or opportunistic infections, and the use of ART, as possible factors contributing to the observed associations. When we examined the mean CD4 counts for the participants in each AF-ALB quartile, we found that the mean was somewhat lower for participants in the highest (fourth) AF-ALB quartile (573±215 cells/µl) compared to participants in AF-ALB quartiles one to three (615±237, 653±280 and 608±247 cells/ml, respectively). However, these means were not significantly different by analysis of variance ($P=0.274$, data not shown). These results seem to indicate that the immune modulating effects of aflatoxin occur early in HIV infection, even well before CD4 cells drop below 500, and that aflatoxin and HIV may act synergistically in impairing the immune system resulting in higher viral loads. Previously, we reported that HIV-positive participants with high AF-ALB had significantly lower percentages of CD4+ Tregs, naive CD4+ T cells and B cells compared to HIV-positive participants with low AF-ALB (Jiang *et al.*, 2008). This could result in increased HIV replication. As expected, both multivariable logistic and linear regression analyses showed that participants with higher CD4 counts had significantly lower viral loads than those with low CD4.

Another possible explanation for the association between high AF-ALB and high HIV viral load, which we discussed in our earlier paper, is that AFB_1 may increase HIV-1

transcription (Jolly *et al.*, 2011). AFB_1, benzo[a]pyrene (BaP) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) have been reported to significantly increase the chloramphenicol acetyltransferase (CAT) reporter gene linked to the promoter sequences in the HIV-1 LTR, thereby increasing the rate of proviral transcription (Yao *et al.*, 1995). TCDD had previously been shown to increase infectious HIV-1 titres in experimental systems (Pokrovsky *et al.*, 1991; Tsyrlov and Pokrovsky, 1993). AFB_1 and BaP were more potent in increasing CAT activity (5-fold) than TCDD (2- to 3-fold) (Yao *et al.*, 1995). Although the mechanism by which AFB_1 increased HIV-1 transcription was not investigated, Yao *et al.* (1995) showed that while TCDD increased CAT expression and HIV transcription in mouse hepatoma Hepa-1 cells, it did not do so in a mutant cell line lacking the CYP1A1 enzyme. They explained that induction of a functional CYP1A1 monooxygenase by TCDD was necessary to stimulate thiol-sensitive reactive oxygen intermediates that are responsible for the TCDD-dependent activation of genes linked to the HIV-1 LTR. AFB_1 is not a polycyclic aromatic hydrocarbon like TCDD and BaP, but it is similar to BaP in that they both are potent carcinogens and both form adducts with guanine in DNA.

Although our study is cross-sectional and limits us in drawing causal associations, the consistent and strong finding that HIV-positive people with higher aflatoxin levels also have higher viral load is substantial and deserves serious consideration. We acknowledge that only aflatoxin was measured in this study and that other mycotoxins, such as fumonisins, which co-occur with aflatoxins in food (Kpodo *et al.*, 2000), may act similarly to aflatoxin. Further studies need to be urgently conducted to understand more about these important toxins and their effects on persons living with HIV and AIDS.

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